Genomic DNA encoding a polypeptide capable of inducing the production of interferon-γ

CROSS-REFERENCE TO RELATED APPLICAIONS

The present application is a division of application no. 09/479,862, filed January 10, 2000, which is a division of application no. 08/884,324, filed June 27, 1997, the entire contents of both applications being hereby incorporated herein by reference.

Background of the Invention

Field of the Invention

The present invention relates to a genomic DNA, more particularly, a genomic DNA encoding a polypeptide capable of inducing the production of interferon-γ (hereinafter abbreviated as "IFN-γ") by immunocompetent cells.

Description of the Prior Art

The present inventors successfully isolated a polypeptide capable of inducing the production of IFN-γ by immunocompetent cells and cloned a cDNA encoding the polypeptide, which is disclosed in Japanese Patent Kokai No.27,189/96 and 193,098/96. Because the present polypeptide possesses the properties of enhancing killer cells' cytotoxicity and inducing killer cells' formation as well as inducing IFN-γ, a useful biologically active protein, it is

expected to be widely used as an agent for viral diseases, microbial diseases, tumors and/or immunopathies, etc.

It is said that a polypeptide generated by a gene expression may be partially cleaved and/or glycosylated by processing with intracellular enzymes in human cells. A polypeptide to be used in therapeutic agents should be preferably processed similarly as in human cells, whereas human cell lines generally have a disadvantage of less producing the present polypeptide, as described in Japanese Patent Application No.269,105/96. Therefore, recombinant DNA techniques should be applied to obtain the present polypeptide in a desired amount. To produce the polypeptide processed similarly as in human cells using recombinant DNA techniques, mammalian cells should be used as the hosts.

Summary of the Invention

In view of foregoing, the first object of the present invention is to provide a DNA which efficiently expresses the polypeptide production when introduced into a mammalian host cell.

The second object of the present invention is to provide a transformant into which the DNA is introduced.

The third object of the present invention is to provide a process for preparing a polypeptide, using the transformant.

[Means to Attain the Object]

The present inventors' energetic studies to attain the above objects succeeded in the finding that a genomic DNA encoding the present polypeptide efficiently expresses the polypeptide production when introduced into mammalian host cells. They found that the polypeptide thus obtained possessed significantly higher biological activities than that obtained by expressing a cDNA encoding the polypeptide in *Escherichia coli*.

The first object of the present invention is attained by a genomic DNA encoding a polypeptide with the amino acid sequence of SEQ ID NO:1 (where the symbol "Xaa" means "isoleucine" or "threonine") or its homologous one, which induces interferon-γ production by immunocompetent cells.

The second object of the present invention is attained by a transformant formed by introducing the genomic DNA into a mammalian host cell.

The third object of the present invention is attained by a process for preparing a polypeptide, which comprises (a) culturing the transformant in a nutrient medium, and (b) collecting the polypeptide from the resultant culture.

Brief Explanation of the Accompanying Drawings

FIG.1 is a restriction map of a recombinant DNA

containing a genomic DNA according to the present invention.

Explanation of the symbols are as follows: The symbol "*Hin* dIII" indicates a cleavage site by a restriction enzyme *Hin* dIII, and the symbol "HuIGIF" indicates a genomic DNA according to the present invention.

Detailed Description of the Invention

The followings are the preferred embodiments according to the present invention. This invention is made based on the identification of a genomic DNA encoding the polypeptide with the amino acid sequence of SEQ ID NO:1 or its homologous one, and the finding that the genomic DNA efficiently expresses the polypeptide with high biological activities when introduced into mammalian host cells. The genomic DNA of the present invention usually contains two or more exons, at least one of which possesses a part of or the whole of the nucleotide sequence of SEQ ID NO:2. The wording "a part" includes a nucleotide and a sequential nucleotides consisting of two or more nucleotides in SEQ ID NO:2. Examples of the exons are SEQ ID NOs:3 and 4. Human genomic DNA may contain additional exons with SEQ ID NOs:5 to 7. Since the present genomic DNA is derived from a mammalian genomic DNA, it contains introns, as a distinctive feature in mammalian genomic DNAs. The present genomic DNA usually has two or more introns such as SEQ ID NOs:8 to 12.

More particular examples of the present genomic DNA include DNAs with SEQ ID NOs:13 and 14 or complementary sequences thereunto. The DNAs with SEQ ID NOs:13 and 14 are substantially the same. The DNA with SEQ ID NO:14 contains coding regions for a leader peptide, consisting of the nucleotides 15,607th-15,685th, 17,057th-17,068th and 20,452nd-20,468th, coding regions for the present polypeptide, consisting of the nucleotides 20,469th-20,586th, 21,921st-22,054th and 26,828th-27,046th, and regions as introns, consisting of the nucleotides 15,686th-17,056th, 17,069-20,451st, 20,587th-21,920th and 22,055th-26,827th. The genomic DNA with SEQ ID NO:13 is suitable for expressing the polypeptide in mammalian host cells.

Generally in this field, when artificially expressing a DNA encoding a polypeptide in a host, one or more nucleotides in a DNA may be replaced by different ones, and appropriate promoter(s) and/or enhancer(s) may be linked to the DNA to improve the expressing efficiency or the properties of the expressed polypeptide. The present genomic DNA can be altered similarly as above. Therefore, as far as not substantially changing in the biological activities of the expressed polypeptides, the present genomic DNA should include DNAs encoding functional equivalents of the polypeptide, formed as follows: One or more nucleotides in SEQ ID NOs:3 to

14 are replaced by different ones, the untranslated regions and/or the coding region for a leader peptide in the 5'- and/or 3'-termini of SEQ ID NOs:3, 4, 5, 6, 7, 13 and 14 are deleted, and appropriate oligonucleotides are linked to either or both ends of SEQ ID NO:13.

The present genomic DNA includes general DNAs which are derived from a genome containing the nucleotide sequences as above, and it is not restricted to its sources or origins as far as it is once isolated from its original organisms. For example, the present genomic DNA can be obtained by chemically synthesizing based on SEQ ID NOs:2 to 14, or by isolating from a human genomic DNA. The isolation of the present genomic DNA from such a human genomic DNA comprises (a) isolating a genomic DNA from human cells by conventional methods, (b) screening the genomic DNA with probes or primers, which are chemically synthesized oligonucleotides with a part of or the whole of the nucleotide sequence of SEQ ID NO:2, and (c) collecting a DNA to which the probes or primers specifically hybridize. Once the present genomic DNA is obtained, it can be unlimitedly replicated by constructing a recombinant DNA with an autonomously replicable vector by conventional method and then introducing the recombinant DNA into an appropriate host such as a microorganism or an animal

cell before culturing the transformant or by applying a PCR method.

The present genomic DNA is very useful in producing the polypeptide by recombinant DNA techniques since it efficiently expresses the polypeptide with high biological activities when introduced into mammalian host cells. The present invention further provides a process for preparing a polypeptide using a specific genomic DNA, comprising the steps of (a) culturing a transformant formed by introducing the present genomic DNA into mammalian host cells, and (b) collecting the polypeptide which induces IFN-γ production by immunocompetent cells from the resultant culture.

The following explains the process for preparing the polypeptide according to the present invention. The present genomic DNA is usually introduced into host cells in the form of a recombinant DNA. The recombinant DNA, comprising the present genomic DNA and an autonomously replicable vector, can be relatively easily prepared by conventional recombinant DNA techniques when the genomic DNA is available. The vectors, into which the present genomic DNA can be inserted, include plasmid vectors such as pcD, pcDL-SRα, pKY4, pCDM8, pCEV4 and pME18S. The autonomously replicable vectors usually further contain appropriate nucleotide sequences for the expression of the present recombinant DNA in each host cell, which include

sequences for promoters, enhancers, replication origins, transcription termination sites, splicing sequences and/or selective markers. Heat shock protein promoters or IFN-α promoters, as disclosed in Japanese Patent Kokai No.163,368/95 by the same applicant of this invention, enables to artificially regulate the present genomic DNA expression by external stimuli.

To insert the present genomic DNA into vectors, conventional methods used in this field can be arbitrarily used: Genes containing the present genomic DNA and autonomously replicable vectors are cleaved with restriction enzymes and/or ultrasonic, and the resultant DNA fragments and the resultant vector fragments are ligated. To cleave genes and vectors by restriction enzymes, which specifically act on nucleotides, more particularly, *Acc*I, *Bam*HI, *Bgl*II, *Bst*XI, *Eco*RI, *Hin*dIII, *Not*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Xba*I, *Xho*I, etc., facilitate the ligation of the DNA fragments and the vector fragments. To ligate the DNA fragments and the vector fragments, they are, if necessary, first annealed, then treated with a DNA ligase *in vivo* or *in vitro*. The recombinant DNAs thus obtained can be unlimitedly replicated in hosts derived from microorganisms or animals.

Any cells conventionally used as hosts in this field can be used as the host cells: Examples of such are

epithelial, interstitial and hemopoietic cells, derived from human, monkey, mouse and hamster, more particularly, 3T3 cells, C127 cells, CHO cells, CV-1 cells, COS cells, HeLa cells, MOP cells and their mutants. Cells which inherently produce the present polypeptide also can be used as the host cells: Example of such are human hemopoietic cells such as lymphoblasts, lymphocytes, monoblasts, monocytes, myeloblasts, myelocytes, granulocytes and macrophages, and human epithelial and interstitial cells derived from solid tumors such as pulmonary carcinoma, large bowel cancer and colon cancer. More particular examples of the latter hemopoietic cells are leukemia cell lines such as HBL-38 cells, HL-60 cells ATCC CCL240, K-562 cells ATCC CCL243, KG-1 cells ATCC CCL246, Mo cells ATCC CRL8066, THP-1 cells ATCC TIB202, U-937 cells ATCC CRL1593.2, described by J. Minowada et al. in "*Cancer Research*", Vol.10, pp.1-18 (1988), derived from leukemias or lymphoma including myelogenous leukemias, promyelocytic leukemias, monocytic leukemias, adult T-cell leukemias and hairy cell leukemias, and their mutants. The present polypeptide-processibility of these leukemia cell lines and their mutants is so distinguished that they can easily yield the polypeptide with higher biological activities when used as hosts.

To introduce the present DNA into the hosts,

conventional methods such as DEAE-dextran method, calcium phosphate transfection method, electroporation method, lipofection method, microinjection method, and viral infection method as using retrovirus, adenovirus, herpesvirus and vaccinia virus, can be used. The polypeptide-producing clones in the transformants can be selected by applying the colony hybridization method or by observing the polypeptide production after culturing the transformants in culture media. For example, the recombinant DNA techniques using mammalian cells as hosts are detailed in "*Jikken-Igaku-Bessatsu Saibo-Kogaku Handbook* (The handbook for the cell engineering)" (1992), edited by Toshio KUROKI, Masaru TANIGUCHI and Mitsuo OSHIMURA, published by YODOSHA. CO., LTD., Tokyo, Japan, and "*Jikken-Igaku-Bessatsu Biomanual Series 3 Idenshi Cloning Jikken-Ho* (The experimental methods for the gene cloning)" (1993), edited by Takahi YOKOTA and Ken-ichi ARAI, published by YODOSHA CO., LTD., Tokyo, Japan.

The transformants thus obtained secrete the present polypeptide intracellularly and/or extracellularly when cultured in culture media. As the culture media, conventional ones used for mammalian cells can be used. The culture media generally comprise (a) buffers as a base, (b) inorganic ions such as sodium ion, potassium ion, calcium ion, phosphoric ion and chloric ion, (c) micronutrients, carbon sources, nitrogen

sources, amino acids and vitamins, which are added depending on the metabolic ability of the cells, and (d) sera, hormones, cell growth factors and cell adhesion factors, which are added if necessary. Examples of individual media include 199 medium, DMEM medium, Ham's F12 medium, IMDM medium, MCDB 104 medium, MCDB 153 medium, MEM medium, RD medium, RITC 80-7 medium, RPMI-1630 medium, RPMI-1640 medium and WAJC 404 medium. The cultures containing the present polypeptide are obtainable by inoculating the transformants into the culture media to give a cell density of 1×10^4 - 1×10^7 cells/ml, more preferably, 1×10^5 - 1×10^6 cells/ml, and then subjecting to suspension- or monolayer-cultures at about 37°C for 1-7 days, more preferably, 2-4 days, while appropriately replacing the culture media with a fresh preparation of the culture media. The cultures thus obtained usually contain the present polypeptide in a concentration of about 1-100 μg/ml, which may vary depending on the types of the transformants or the culture conditions used.

While the cultures thus obtained can be used intact as an IFN-γ inducer, they are usually subjected to a step for separating the present polypeptide from the cells or the cell debris using filtration, centrifugation, etc. before use, which may follow a step for disrupting the cells with supersonication, cell-lytic enzymes and/or detergents if

desired, and to a step for purifying the polypeptide. The cultures from which the cells or cell debris are removed are usually subjected to conventional methods used in this field for purifying biologically active polypeptides, such as salting-out, dialysis, filtration, concentration, separatory sedimentation, ion-exchange chromatography, gel filtration chromatography, adsorption chromatography, chromatofocusing, hydrophobic chromatography, reversed phase chromatography, affinity chromatography, gel electrophoresis and/or isoelectric focusing. The resultant purified polypeptide can be concentrated and/or lyophilized into liquids or solids depending on final uses. The monoclonal antibodies disclosed in Japanese Patent Kokai No.231,598/96 by the same applicant of this invention are extremely useful to purify the present polypeptide. Immunoaffinity chromatography using monoclonal antibodies yields the present polypeptide in a relatively high purity at the lowest costs and labors.

The polypeptide obtainable by the process according to the present invention exerts strong effects in the treatment and/or the prevention for IFN-γ- and/or killer cell-susceptive diseases since it possesses the properties of enhancing killer cells' cytotoxicity and inducing killer cells' formation as well as inducing IFN-γ, a useful biologically active protein, as described above. The

polypeptide according to the present invention has a high activity of inducing IFN-γ, and this enables a desired amount of IFN-γ production with only a small amount. The polypeptide is so low toxic that it scarcely causes serious side effects even when administered in a relatively-high dose. Therefore, the polypeptide has an advantage that it can readily induce IFN-γ in a desired amount without strictly controlling the dosage. The uses as agents for susceptive diseases are detailed in Japanese Patent Application No.28,722/96 by the same applicant of this invention.

The present genomic DNA is also useful for so-called "gene therapy". According to conventional gene therapy, the present DNA can be introduced into patients with IFN-γ- and/or killer cell-susceptive diseases by directly injecting after the DNA is inserted into vectors derived from viruses such as retrovirus, adenovirus and adeno-associated virus or is incorporated into cationic- or membrane fusible-liposomes, or by self-transplanting lymphocytes which are collected from patients before the DNA is introduced. In adoptive immunotherapy with gene therapy, the present DNA is introduced into effector cells similarly as in conventional gene therapy. This can enhance the cytotoxicity of the effector cells to tumor cells, resulting in improvement of the adoptive immunotherapy. In tumor vaccine therapy with gene therapy,

tumor cells from patients, into which the present genomic DNA is introduced similarly as in conventional gene therapy, are self-transplanted after proliferated *ex vivo* up to give a desired cell number. The transplanted tumor cells act as vaccines in the patients to exert a strong antitumor immunity specifically to antigens. Thus, the present genomic DNA exhibits considerable effects in gene therapy for diseases including viral diseases, microbial diseases, malignant tumors and immunopathies. The general procedures for gene therapy are detailed in "*Jikken-Igaku-Bessatsu Biomanual UP Series Idenshichiryo-no-Kisogijutsu* (Basic techniques for the gene therapy)" (1996), edited by Takashi ODAJIMA, Izumi SAITO and Keiya OZAWA, published by YODOSHA CO., LTD., Tokyo, Japan.

The following examples explain the present invention, and the techniques used therein are conventional ones used in this field: For example, the techniques are described in "*Jikken-Igaku-Bessatsu Saibo-Kogaku Handbook* (The handbook for the cell engineering)", (1992), edited by Toshio KUROKI, Masaru TANIGUCHI and Mitsuo OSHIMURA, published by YODOSHA CO., LTD., Tokyo, Japan, and "*Jikken-Igaku-Bessatsu Biomanual Series 3 Idenshi Clonong Jikken-Ho* (The experimental methods for the gene cloning)" (1993), edited by Takahi YOKOTA and Ken-ichi ARAI, published by YODOSHA CO., LTD., Tokyo, Japan.

Example 1

Cloning genomic DNA and determination of nucleotide sequence

Example 1-1

Determination of partial nucleotide sequence

Five ng of "PromoterFinder™ DNA PvuII LIBRARY", a human placental genomic DNA library commercialized by CLONTECH Laboratories, Inc., California, USA, 5 μl of 10 x Tth PCR reaction solution, 2.2 μl of 25 mM magnesium acetate, 4 μl of 2.5 mM dNTP-mixed solution, one μl of the mixed solution of 2 unit/μl rTth DNA polymerase XL and 2.2 μg/μl Tth Start Antibody in a ratio of 4:1 by volume, 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-CCATCCTAA TACGACTCACTATAGGGC-3' (SEQ ID NO:16) as an adaptor primer, and 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-TTCCTCTTCCCGAAGCTGTGTAGACTGC-3' (SEQ ID NO:17) as an anti-sense primer, which was chemically synthesized based on the sequence of the nucleotides 88th-115th in SEQ ID NO:2, were mixed and volumed up to 50 μl with sterilized distilled water. After incubating at 94°C for one min, the mixture was subjected to 7 cycles of incubations at 94°C for 25 sec and at 72°C for 4 min, followed by 32 cycles of incubations at 94°C for 25 sec at 67°C for 4 min to perform PCR.

The reaction mixture was diluted by 100 folds with sterilized distilled water. One μl of the dilution, 5 μl of

10 x Tth PCR reaction solution, 2.2 μl of 25 mM magnesium acetate, 4 μl of 2.5 mM dNTP-mixed solution, one μl of the mixed solution of 2 unit/μl rTth DNA polymerase XL and 2.2 μg/μl Tth Start Antibody in a ratio of 4:1 by volume, 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-CTA TAGGGCACGCGTGGT-3' (SEQ ID NO:18) as a nested primer, and 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-TTCCTCTTCCCGAAGCTGTGTAGACTGC-3' (SEQ ID NO:19) as an anti-sense primer, which was chemically synthesized similarly as above, were mixed and volumed up to 50 μl with sterilized distilled water. After incubating at 94°C for one min, the mixture was subjected to 5 cycles of incubations at 94°C for 25 sec and at 72°C for 4 min, followed by 22 cycles of incubations at 94°C for 25 sec and at 67°C for 4 min to perform PCR for amplifying a DNA fragment of the present genomic DNA. The genomic DNA library and reagents for PCR used above were mainly from "PromoterFinder™ DNA WALKING KITS", commercialized by CLONTECH Laboratories, Inc., California, USA

An adequate amount of the PCR product thus obtained was mixed with 50 ng of "pT7 Blue(R)", a plasmid vector commercialized by Novagen, Inc., WI, USA, and an adequate amount of T4 DNA ligase, and 100 mM ATP was added to give a final concentration of one mM, followed by incubating at 16°C for 18 hr to insert the DNA fragment into the plasmid vector.

The obtained recombinant DNA was introduced into an *Escherichia coli* JM109 strain by the competent cell method to form a transformant, which was then inoculated into L-broth medium (pH 7.2) containing 50 μg/ml ampicillin and cultured at 37°C for 18 hr. The cells were isolated from the resulting culture, and then subjected to the conventional alkali-SDS method to collect a recombinant DNA. The dideoxy method analysis confirmed that the recombinant DNA contained the DNA fragment with a sequence of the nucleotides 5,150th-6,709th in SEQ ID NO:14.

Example 1-2

Determination of partial nucleotide sequence

PCR was performed in the same conditions as the first PCR in Example 1-1, but an oligonucleotide with the nucleotide sequence of 5'-GTAAGTTTTCACCTTCCAACTGTAGAGTCC-3' (SEQ ID NO:20), which was chemically synthesized based on the nucleotide sequence of the DNA fragment in Example 1-1, was used as an anti-sense primer.

The reaction mixture was diluted by 100 folds with sterilized distilled water. One μl of the dilution was placed into a reaction tube, and PCR was performed in the same conditions as used in the second PCR in Example 1-1 to amplify another DNA fragment of the present genomic DNA, but an oligonucleotide with the nucleotide sequence of 5'-GGGATCAAGT

AGTGATCAGAAGCAGCACAC-3' (SEQ ID NO:21), which was chemically synthesized based on the nucleotide sequence of the DNA fragment in Example 1-1, was used as an anti-sense primer.

The DNA fragment was inserted into the plasmid vector similarly as in Example 1-1 to obtain a recombinant DNA. The recombinant DNA was replicated in *Escherichia coli* before being collected. The analysis of the collected recombinant DNA confirmed that it contained the DNA fragment with a sequence of the nucleotides 1st-5, 228th in SEQ ID NO:14.

Example 1-3

Determination of partial nucleotide sequence

0.5 μg of a human placental genomic DNA, commercialized by CLONTECH Laboratories, Inc., California, USA, 5 μl of 10 x PCR reaction solution, 8 μl of 2.5 mM dNTP-mixed solution, one μl of the mixed solution of 5 unit/μl "TAKARA LA Taq POLYMERASE" and 1.1 μg/μl "TaqStart ANTIBODY" in a ratio of 1:1 by volume, both of them are commercialized by Takara Syuzo Co., Tokyo, Japan, 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-CCTGGCTG CCAACTCTGGCTGCTAAAGCGG-3' (SEQ ID NO:22) as a sense primer, chemically synthesized based on a sequence of the nucleotides 46th-75th in SEQ ID NO:2, and 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-GTATTGTCAATAAATTTCATTGC

CACAAAGTTG-3' (SEQ ID NO:23) as an anti-sense primer, chemically synthesized based on a sequence of the nucleotides 210th-242nd in SEQ ID NO:2, were mixed and volumed up to 50 μl with sterilized distilled water. After incubating at 94°C for one min, the mixture was subjected to 5 cycles of incubations at 98°C for 20 sec and at 68°C for 10 min, followed by 25 cycles of incubations at 98°C for 20 sec and 68°C for 10 min, with adding 5 sec in times to every cycle, and finally incubated at 72°C for 10 min to amplify further DNA fragment of the present genomic DNA. The reagents for PCR used above were mainly from "TAKARA LA PCR KIT VERSION 2", commercialized by Takara Syuzo Co., Tokyo, Japan.

The DNA fragment was inserted into the plasmid vector similarly as in Example 1-1 to obtain a recombinant DNA. The recombinant DNA was replicated in *Escherichia coli* before being collected. The analysis of the collected recombinant DNA confirmed that it contained the DNA fragment with a sequence of the nucleotides 6,640th-15,671st in SEQ ID NO:14.

Experiment 1-4

Determination of partial nucleotide sequence

PCR was performed in the same conditions as the PCR in Example 1-3 to amplify further another DNA fragment of the present genomic DNA; but an oligonucleotide with the

nucleotide sequence of 5'-AAGATGGCTGCTGAACCAGTAGAAGACAATTGC-3' (SEQ ID NO:24), chemically synthesized based on a sequence of the nucleotide 175th-207th in SEQ ID NO:2, was used as a sense primer, an oligonucleotide with the nucleotide sequence of 5'-TCCTTGGTCAATGAAGAGAACTTGGTC-3' (SEQ ID NO:25), chemically synthesized based on a sequence of nucleotides 334th-360th in the SEQ ID NO:2, was used as an anti-sense primer, and after incubating at 98°C for 20 sec, the reaction mixture was subjected to 30 cycles of incubations at 98°C for 20 sec and at 68°C for 3 min, followed by incubating at 72°C for 10 min.

The DNA fragment was inserted into the plasmid vector similarly as in Example 1-1 to obtain a recombinant DNA. The recombinant DNA was replicated in *Escherichia coli* before being collected. The analysis of the collected recombinant DNA confirmed that it contained the DNA fragment with a sequence of the nucleotides 15,604th-20,543rd in SEQ ID NO:14.

Example 1-5

Determination of partial nucleotide sequence

PCR was performed in the same conditions as the PCR in Example 1-4 to amplify further another DNA fragment of the present genomic DNA, but an oligonucleotide with the nucleotide sequence of 5'-CCTGGAATCAGATTACTTTGGCAAGCTTGAATC-3' (SEQ ID NO:26), chemically synthesized based on the sequence

of the nucleotide 273rd-305th in SEQ ID NO:2, was used as a sense primer, and an oligonucleotide with the nucleotide sequence of 5'-GGAAATAATTTTGTTCTCACAGGAGAGAGTTG-3' (SEQ ID NO:27), chemically synthesized based on the sequence of nucleotides 500th-531st in the SEQ ID NO:2, was used as an anti-sense primer.

The DNA fragment was inserted into the plasmid vector similarly as in Example 1-1 to obtain a recombinant DNA. The recombinant DNA was replicated in *Escherichia coli* before being collected. The analysis of the collected recombinant DNA confirmed that it contained the DNA fragment with a sequence of the nucleotides 20,456th-22,048th in SEQ ID NO:14.

Example 1-6

Determination of partial nucleotide sequence

PCR was performed in the same conditions as the PCR in Example 1-4 to amplify further another DNA fragment of the present genomic DNA, but an oligonucleotide with the nucleotide sequence of 5'-GCCAGCCTAGAGGTATGGCTGTAACTATCTC-3' (SEQ ID NO:28), chemically synthesized based on the sequence of the nucleotide 449th-479th in SEQ ID NO:2, was used as a sense primer, and an oligonucleotide with the nucleotide sequence of 5'-GGCATGAAATTTTAATAGCTAGTCTTCGTTTTG-3' (SEQ ID NO:29), chemically synthesized based on the sequence of

nucleotides 745th-777th in the SEQ ID NO:2, was used as an anti-sense primer.

The DNA fragment was inserted into the plasmid vector similarly as in Example 1-1 to obtain a recombinant DNA. The recombinant DNA was replicated in *Escherichia coli* before being collected. The analysis of the collected recombinant DNA confirmed that it contained the DNA fragment with a sequence of the nucleotides 21,996th-27,067th in SEQ ID NO:14.

Example 1-7

Determination of partial nucleotide sequence

PCR was performed in the same conditions as the first PCR in Example 1-2 to amplify further another DNA fragment in the present genomic DNA, but an oligonucleotide with the nucleotide sequence of 5'-GTGACATCATATTCTTTCAGA GAAGTGTCC-3' (SEQ ID NO:30), chemically synthesized based on the sequence of the nucleotide 575th-604th in SEQ ID NO:2, was used as a sense primer.

The reaction mixture was diluted by 100 folds with sterilized distilled water. One μl of the dilution was placed into a reaction tube, and PCR was performed in the same conditions as the second PCR in Example 1-2 to amplify further another DNA fragment of the present genomic DNA, but an

oligonucleotide with the sequence of 5'-GCAATTTGAATCTTCATC ATACGAAGGATAC-3' (SEQ ID NO:31), chemically synthesized based on a sequence of the nucleotides 624th-654th in SEQ ID NO:2, was used as a sense primer.

The DNA fragment was inserted into the plasmid vector similarly as in Example 1-1 to obtain a recombinant DNA. The recombinant DNA was replicated in *Escherichia coli* before being collected. The analysis of the collected recombinant DNA confirmed that it contained the DNA fragment with a sequence of the nucleotides 26,914th-28,994th in SEQ ID NO:14.

Example 1-8

Determination of complete nucleotide sequence

Comparing the nucleotide sequence of SEQ ID NO:2, which was proved to encode the present polypeptide, as disclosed in Japanese Patent Kokai No.193,098/96 by the same applicant of this invention, with the partial nucleotide sequences identified in Examples 1-1 to 1-7, it was proved that the present genomic DNA contained the nucleotide sequence of SEQ ID NO:14. SEQ ID NO:14, consisting of 28,994 base pairs (bp), was extremely longer than the SEQ ID NO:2, consisting of only 471 bp. This suggested that SEQ ID NO:14 contained introns, a characteristic of eukaryotic cells.

It was examined where partial nucleotide sequences of SEQ ID NO:2, i.e., exons, and the donor and acceptor sites in introns, respectively consisting of the nucleotides of GT and AG, located in SEQ ID NO:14. Consequently, it was proved that SEQ ID NO:14 contained at least 5 introns, which located in the order of SEQ ID NOs:10, 11, 12, 8 and 9 in the direction from the 5'- to the 3'-termini. Therefore, the sequences between the neighboring introns must be exons, which were thought to be located in the order of SEQ ID NOs:5, 6, 3, 4 and 7 in the direction from the 5'- to the 3'-termini. It was also proved that SEQ ID NO:7 contained the 3'-untranslated region other than the exons. The features of the sequence elucidated as this are arranged in SEQ ID NO:14.

As disclosed in Japanese patent application by the same applicant of this invention, the present polypeptide is produced as a polypeptide with N-terminal amino acid of tyrosine other than methionine in human cells, which is observed in SEQ ID NO:1. This suggests that the present genomic DNA contains a leader peptide region in the upstream of the 5'-terminus of the present polypeptide-encoding region. A sequence consisting of 36 amino acids encoded by the upstream of the nucleotides 20,469th-20,471st. which is the nucleotides of TAC, are described as a leader peptide in SEQ ID NO:14.

Example 2

Preparation of recombinant DNA pBGHuGF for expression

0.06 ng of the DNA fragment in Example 1-4 in a concentration of 3 ng/50 μl, 0.02 ng of the DNA fragment, obtained by the methods in Example 1-5, 5 μl of 10 x LA PCR reaction solution, 8 μl of 2.5 mM dNTP-mixed solution, one μl of the mixed solution of 5 unit/μl TAKARA LA Taq polymerase and 1.1 μg/μl TaqStart Antibody in a ratio of 1:1 by volume, 10 pmol of an oligonucleotide with the sequence of 5'-TCC GAAGCTTAAGATGGCTGCTGAACCAGTA-3' (SEQ ID NO:32) as a sense primer, chemically synthesized based on the nucleotide sequence of the DNA fragment in Example 1-4, and 10 pmol of an oligonucleotide with the nucleotide sequence of 5'-GGAAATAA TTTTGTTCTCACAGGAGAGAGTTG-3' (SEQ ID NO:33) as an anti-sense primer, chemically synthesized based on the nucleotide sequence of the DNA fragment in Example 1-5, were mixed and volumed up to 50 μl with sterilized distilled water. After incubating at 94°C for one min, the mixture was subjected to 5 cycles of incubations at 98°C for 20 sec and at 72°C for 7 min, followed by 25 cycles of incubations at 98°C for 20 sec and 68°C for 7 min to perform PCR. The reaction mixture was cleaved by restriction enzymes *Hind*III and *Sph*I to obtain a DNA fragment of about 5,900 bp, with cleavage sites by *Hind*III and *Sph*I in its both termini.

PCR was performed in the same condition as above, but 0.02 ng of the DNA fragment in Example 1-5, 0.06 ng of the DNA fragment obtained in Example 1-6, an oligonucleotide with the nucleotide sequence of 5'-ATGTAGCGGCCGCGGCATGAAATTTTAA TAGCTAGTC-3' (SEQ ID NO:34) as an anti-sense primer, chemically synthesized based on the nucleotide sequence of the DNA fragment in Example 1-6, and an oligonucleotide with the sequence of 5'-CCTGGAATCAGATTACTTTGGCAAGCTTGAATC-3' (SEQ ID NO:35) as a sense primer, chemically synthesized based on the DNA fragment in Example 1-6, were used. The reaction mixture was cleaved by restriction enzymes *Not*I and *Sph*I to obtain a DNA fragment of about 5,600 bp, with cleavage sites by *Not*I and *Sph*I in its both termini.

A plasmid vector "pRc/CMV", containing a *cytomegalovirus* promoter, commercialized by Invitrogen Corporation, San Diego, USA, was cleaved by restriction enzymes *Hin*dIII and *Not*I to obtain a vector fragment of about 5,500 bp. The vector fragment was mixed with the above two DNA fragments of about 5,900 bp and 5,600 bp, and reacted with T4 DNA ligase to insert the two DNA fragments into the plasmid vector. An *Escherichia coli* JM109 strain was transformed with the obtained recombinant DNA, and the transformant with the plasmid vector was selected by the colony hybridization method. The selected recombinant DNA was named as "pBGHuGF".

As shown in FIG.1, the present genomic DNA, with the nucleotide sequence of SEQ ID NO:13, was ligated in the

downstream of the cleavage site by the restriction enzyme *Hin*dIII in the recombinant DNA.

Example 3

Preparation of transformant using CHO cell as host

CHO-K1 cells ATCC CCL61 were inoculated into Ham's F12 medium (pH 7.2) containing 10 v/v % bovine fetal serum and proliferated by conventional manner. The proliferated cells were collected and washed with phosphate-buffered saline (hereinafter abbreviated as "PBS") followed by suspending in PBS to give a cell density of 1 x 10^7 cells/ml.

10 μg of the recombinant DNA pBGHuGF in Example 2 and 0.8 ml of the above cell suspension were placed in a cuvette and ice-chilled for 10 min. The cuvette was installed in "GENE PULSER", an electroporation device commercialized by Bio-Rad Laboratories Inc., Brussels, Belgium, and then pulsed once with an electric discharge. After pulsing, the cuvette was immediately took out and ice-chilled for 10 min. The cell suspension from the cuvette was inoculated into Ham's F12 medium (pH 7.2) containing 10 v/v % bovine fetal serum and cultured under an ambient condition of 5 v/v % CO_2 at 37°C for 3 days. To the culture medium was added G-418 to give a final

concentration of 400 μg/ml, and the culturing was continued further 3 weeks under the same conditions. From about 100 colonies formed, 48 colonies were selected, and a portion of each was inoculated into a well of culturing plates with Ham's F12 medium (pH7.2) containing 400 μg/ml G-418 and 10 v/v % bovine fetal serum and cultured similarly as above. Thereafter, to each well of the culturing plates was added 10 mM Tris-HCl buffer (pH 8.5) containing 5.1 mM magnesium chloride, 0.5 w/v % sodium deoxycholate, 1 w/v % NONIDET P-40, 10 μg/ml aprotinin and 0.1 w/v % SDS to lyse the cells.

50 μl aliquot of the cell lysates was mixed with one ml of glycerol and incubated at 37°C for one hour, before the polypeptides in the cell lysates were separated by the SDS-polyacrylamide gel electrophoresis. The separated polypeptides were transferred to a nitrocellulose membrane in usual manner, and the membrane was soaked in the culture supernatant of the hybridoma H-1, disclosed in Japanese Patent Kokai No.231,598/96 by the same applicant of this invention, followed by washing with 50 mM Tris-HCl buffer containing 0.05 v/v % TWEEN 20 to remove an excessive mount of the monoclonal antibody. Thereafter, the nitrocellulose membrane was soaked in PBS containing rabbit-derived anti-mouse immunoglobulin antibody for one hr, which was labeled with horseradish peroxidase, followed by washing 50 mM Tris-HCl buffer (pH 7.5)

containing 0.05 v/v % TWEEN 20 and soaking in 50 mM Tris-HCl buffer (pH 7.5) containing 0.005 v/v % hydrogen peroxide and 0.3 mg/ml diaminobenzidine to develop colorations. The clone, which highly produced the polypeptide, was selected based on the color development and named "BGHuGF".

Example 4

Production of polypeptide by transformant and its physicochemical properties

The transformant BGHuGF in Experiment 3 was inoculated into Ham's F12 medium (pH 7.2) containing 400 μg/ml G-418 and 10 v/v % bovine fetal serum, and cultured under an ambient condition of 5 v/v % CO_2 at 37°C for one week. The proliferated cells were collected, washed with PBS, and then washing with 10-fold volumes of ice-chilled 20 mM Hepes buffer (pH 7.4), containing 10 mM potassium chloride and 0.1 mM ethylendiaminetetraacetate bisodium salt, according to the method described in "*Proceedings of The National Academy of The Sciences of The USA*", vol.86, pp.5,227-5,231 (1989), by M. J. Kostura et al. The cells thus obtained were allowed to stand in 3-fold volumes of a fresh preparation of the same buffer under an ice-chilling condition for 20 min and freezed at -80°C, succeeded by thawing to disrupt the cells. The resulting cells were centrifuged to collect the supernatant.

In parallel, THP-1 cells ATCC TIB 202, derived from

a human acute monocytic leukemia, was similarly cultured and disrupted. Supernatant, obtained by centrifuging the resulting cells, was mixed with the supernatant obtained from the transformant BGHuGF and incubated at 37°C for 3 hr to react. The reaction mixture was applied to a column with "DEAE-SEPHAROSE", a gel for ion-exchange chromatography, commercialized by Pharmacia LKB Biotechnology AB, Upsalla, Sweden, equilibrated with 10 mM phosphate buffer (pH 6.6) before use. After washing the column with 10 mM phosphate buffer (pH 6.6), 10 mM phosphate buffer (pH 6.6) with a stepwise gradient of NaCl increasing from 0 M to 0.5 M was fed to the column, and fractions eluted by about 0.2 M NaCl were collected. The fractions were dialyzed against 10 mM phosphate buffer (pH 6.8) before applied to a column with "DEAE 5PW", a gel for ion-exchange chromatography, commercialized by TOSOH Corporation, Tokyo, Japan. To the column was fed 10 mM phosphate buffer (pH 6.8) with a linear gradient of NaCl increasing from 0 M to 0.5 M, and fractions eluted by about 0.2-0.3 M NaCl were collected.

While the obtained fractions were pooled and dialyzed against PBS, a gel for immunoaffinity chromatography with the monoclonal antibody were prepared according to the method disclosed in Japanese Patent Kokai No.231,598/96 by the same applicant of this invention. After the gel were charged

into a plastic column and washed with PBS, the above dialyzed solution was applied to the column. To the column was fed 100 mM glycine-HCl buffer (pH 2.5), and the eluted fractions, which contained a polypeptide capable of inducing the production of IFN-γ by immunocompetent cells, were collected. After the collected fractions were dialyzed against sterilized distilled water and concentrated with a membrane filtration, the resultant was lyophilized to obtain a purified solid polypeptide in a yield of about 15 mg/l-culture.

Example for Reference

Expression in Escherichia coli

As disclosed in Japanese Patent Kokai No.193,098/96, a transformant pKHuGF which was obtained by introducing a cDNA with the nucleotide sequence of SEQ ID NO:2 into Escherichia coli as a host, was inoculated into L-broth medium containing 50 μg/ml ampicillin and cultured at 37°C for 18 hr under shaking conditions. The cells were collected by centrifuging the resulting culture, and then suspended in a mixture solution (pH 7.2) of 139 mM NaCl, 7 mM NaH_2PO_4 and 3 mM Na_2HPO_4, followed by supersonicating to disrupt the cells. After the cell disruptants were centrifuged, the supernatant was subjected to purifying steps similarly as in Example 4-1 to obtain a purified solid polypeptide in a yield of about 5 mg/l-culture.

Comparing the yields of the polypeptides in Example for Reference and in Example 4-1 shows that the use of a transformant, which is formed by introducing a genomic DNA encoding the present polypeptide into a mammalian cell as a host, strongly elevates the yield of the polypeptide per culture.

Example 4-2

Physicochemical property of polypeptide

Example 4-2(a)

Biological activity

Blood were collected from a healthy donor by using a syringe containing heparin, and then diluted with 2-fold volume of serum-free RPMI-1640 medium (pH 7.4). The blood was overlaid on ficoll, commercialized by Pharmacia LKB Biotechnology AB, Upsalla, Sweden, and centrifuged to obtain lymphocytes, which were then washed with RPMI-1640 medium containing 10 v/v % bovine fetal serum before being suspended in a fresh preparation of the same medium to give a cell density of 5×10^6 cells/ml. 0.15 ml aliquots of the cell suspension was distributed into wells of micro plates with 96 wells.

To the wells with the cells were distributed 0.05 ml aliquots of solutions of the polypeptide in Example 4-1, diluted with RPMI-1640 medium (pH 7.4) containing 10 v/v %

bovine fetal serum to give desired concentrations. 0.05 ml aliquots of fresh preparations of the same medium with 2.5 μg/ml concanavalin A were further added to the wells, before culturing in a 5 v/v % CO_2 incubator at 37°C for 24 hr. After the cultivation, 0.1 ml of the culture supernatant was collected from each well and examined on IFN-γ by usual enzyme immunoassay. In parallel, a systems as a control using the polypeptide in Reference for that in Example 4-1 or using no polypeptide was treated similarly as above. The results were in Table 1. IFN-γ in Table 1 were expressed with international units (IU), calculated based on the IFN-γ standard, Gg23-901-530, obtained from the International Institute of Health, USA

Table 1

Sample of polypeptide	IFN-γ production (IU/ml)
Example 4-2(a)	3.4×10^5
Example for Reference	1.7×10^5

Table 1 indicates that the lymphocytes as immunocompetent cells produce IFN-γ by the action of the present polypeptide.

It is more remarkable that the polypeptide in Example 4-1 could induce IFN-γ production more than that in

Example for Reference. Considering this and the difference in the yields of the polypeptides, described in Example for Reference, it can be presumed: Even if DNAs could be substantially equivalent in encoding the same amino acid sequence, not only the expressing efficiencies of the DNAs may differ, but the products expressed by them may significantly differ in their biological activities as a result of post-translational modifications by intracellular enzymes, depending on types of the DNAs and their hosts; (a) one type is used a transformant formed by introducing a DNA, which is a cDNA, into a microorganisms as a host, and (b) other type is used a transformant formed by introducing the present genomic DNA into a mammalian cell as a host.

Example 4-2(b)

Molecular weight

SDS-polyacrylamide gel electrophoresis of the polypeptide in Example 4-1 in the presence of 2 w/v % dithiothreitol as a reducing agent, according to the method reported by U. K. Laemli et al., in "Nature", Vol.227, pp.680-685 (1970), exhibited a main band of a protein capable of inducing IFN-γ in a position corresponding to a molecular weight of about 18,000-19,500 daltons. The molecular weight makers used in the analysis were bovine serum albumin (67,000 daltons), ovalbumin (45,000 daltons), carbonic anhydrase

(30,000 daltons), soy bean trypsin inhibitor (20,100 daltons) and α-lactoalbumin (14,000 daltons).

Example 4-2(c)

N-Terminal amino acid sequence

Conventional analysis using "MODEL 473A", a protein sequencer commercialized by Perkin-Elmer Corp., Norwalk, USA, revealed that the polypeptide in Example 4-1 had the amino acid sequence of SEQ ID NO:15 in the N-terminal region.

Judging collectively from this result as well as the information that SDS-polyacrylamide gel electrophresis exhibited a main band in a position corresponding to a molecular weight of about 18,000-19,500 daltons, and that the molecular weight calculated from the amino acid sequence of SEQ ID NO:1 was 18,199 daltons, it can be concluded that the polypeptide in Example 4-1 has the amino acid sequence of SEQ ID NO:6.

As is described above, the present invention is made based on the identification of a genomic DNA encoding the polypeptide which induces the production of IFN-γ by immunocompetent cells. The present genomic DNA efficiently express the present polypeptide when introduced into mammalian host cells. The polypeptide features higher biological activities than that obtained by the cDNA expression in

Escherichia coli. Therefore, the present genomic DNA is useful for the recombinant DNA techniques to prepare the polypeptide capable of inducing IFN-γ production by immunocompetent cells. The present genomic DNA is useful to gene therapy for diseases including viral diseases, bacterial-infectious diseases, malignant tumors and immunopathies.

Thus, the present invention is a significant invention which has a remarkable effect and gives a great contribution to this field.

While there has been described what is at present considered to be the preferred embodiments of the present invention, it will be understood the various modifications may be made therein, and it is intended to cover in the appended claims all such modifications as fall within the true spirits and scope of the invention.